Mem. Inst. Butantan,
30:133-142. 1960-62.

HEMOLYTIC ACTIVITY OF ANIMAL VENOMS. II. VARIATION IN RELATION TO ERYTHROCYTE SPECIES. (*)

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Sensitivity variations of erythrocytes from different animal species to hemolysis have called the attention of many research workers during experiments on hemolysis provoked by snake venoms. Some of them have only pointed out this variability, such as Mitchell and Reichert (12) who found in 1886 that bird erythrocytes are more resistant than those of Mammalia. Pestana (14), in 1908, and Brazil and Pestana (3), in 1910, found differences in sensitivity among the red blood cells of ox, rabbit, guinea-pig, horse and rat. Other research workers made comparative investigations on the behaviour of different erythrocyte types, submitted either to the action of one special venom, as described by Kyes (10,11) in 1902 and 1910, Phisalix (15) in 1903 and Ganguly (5) in 1937, or to the action of several venoms as described by Flexner and Noguchi (4) in 1902, Houssay and Negrete (8) in 1922 and Kellaway and Williams (9) in 1933.

Differences in results already existing with regard to the classification of snake venoms according to their hemolytic potency (14, 3, 8, 13) could be chiefly attributed to the fact that each of them is refered to different erythrocytes.

Spider venoms studied by Walbum (18) and Houssay (7) have not been considered as hemolytic. But hemolysins were found in body extracts of these animals (18) or in the eggs of some species (7). Variations in sensitivity of different red cells were also observed in relation to these hemolysins.

The experiments reported in this paper were made in order to find out if some of the conflicting findings reported in the older literature could be due to the use of erythrocytes from different species, and diversity of methods. For this purpose a comparison was made concerning the indirect hemolytic activity of different animal venoms regarding various erythrocyte species. Some experiments were also done to analyse the influence of deep-freezer storing on the ability of horse serum to act as lecithin source in hemolysis tests. Quantitative

(*) This work was supported by an aid of the Research Fund of Instituto Butantan (FPIB).
(**) Fellow of the Research Fund of Instituto Butantan.

techniques have been used with standardization of each stage of the method in order to permit further comparative investigations. By this way a real evaluation of the different sensitivities of erythrocytes could be carried out as well as a comparison among the different venoms utilized.

MATERIAL AND METHODS

Saline solution — Samples of venom were suspended in a saline solution prepared as described previously (17).

Venom — Experiments were carried out with dried venoms of snakes (*Elapinae, Viperinae, Crotalinae*), spiders (*Theraphosinae, Grammostolinae, Cteninae, Lycosinae* and *Sicariinae*) and scorpions (*Centurinae*) from the stock of Instituto Butantan. Solutions of 1mg/ml in the above described saline, were prepared just before use. The whole series of venoms was tested in the same day on the same type of erythrocytes. Venoms collected in Instituto Butantan were vacuum-dried and stored in the dark, at room temperature.

Serum — Lecithin for testing the hemolytic activity of venoms was supplied from pooled horse sera, inactivated at 55°C for 30 minutes, and kept at —15°C. For dog red cells, however, homologous serum was added since these erythrocytes were hemolyzed by horse serum.

Erythrocytes — Human blood and that of rhesus, horse, ox, sheep, dog, rabbit, guinea-pig, rat and mouse were collected in Alsever solution and kept at +4°C. The erythrocytes were always utilized at the 4th day after being collected. They were centrifuged and washed 4 times with 0,85% NaCl (w/v) before a suspension was prepared containing 20 million erythrocytes per ml. One ml of the suspension was hemolyzed by 4 ml of distilled water, and, after centrifugation, the liberated hemoglobin was determined by measuring the absorption of solutions at 540 mμ, according to the technique standardized by Rosenfeld, Kelen and Nudel (17). The absorption value obtained (65 to 76% depending upon the species of the erythrocytes) was taken to correspond to 100% hemolysis.

Test — For the determinations of erythrocytes sensitivity of different animal species to the indirect activity of venoms, 1 ml of serum was added to 1 ml red cells with constant concentration of $20x10^6$; afterwards 1 mg of venom in 1 ml of the saline solution was added. The volume was made up to 5 ml and the mixture was allowed to stand 60 minutes in a water-bath at 37°C and then transfered to an ice water-bath while centrifugation took place in order to separate cells and stroma of the lyzed cells. From the light absorption of the supernatant, the amount of hemoglobin liberated was obtained and thereto the number of corpuscles hemolyzed by the venom. The hemolysis produced by the venoms was indicated in percentage of liberated hemoglobin in relation to the 100% standard. All tests were carried out in duplicate.

RESULTS

Table 1 and 2 refer to percentages of hemolysis produced by venoms on erythrocytes from different species. Venoms are grouped according to family, sub-family, genus and species in order to give a clearer interpretation of data. Only indirect hemolysis has been stated. Attention should be called to the fact that horse serum had hemolytic activity on dog erythrocytes, what can be a cause of error.

SNAKE VENOMS — Almost all snake venoms used presented hemolytic activity on al of the 10 erythrocyte species, some of them being more or less sensitive to the hemolysin. However, two venoms, belonging to different families, *Bothrops cotiara* and *Micrurus frontalis* were exceptions; the venom of *M. frontalis* did not hemolyze human or ox red cells, while the venom of *B. cotiara* did not hemolyze the erythrocytes of human, ox, sheep, rabbit or rhesus monkey.

The mean hemolysis values in relation to all venoms (table 1) indicate the sensitivity degree as follows: horse 89%; rhesus 88%; rabbit 87%; human 85%; rat 84%; guinea-pig 77%; dog 73%; ox 70%; mouse 66%; sheep 59%.

TABLE 1 — Percentage of hemolysis provoked by venoms on red blood cells of different species

1 mg of venom — 20 x 10^6 red blood cells

SNAKES	Venom origin	Human	Rhesus	Dog	Horse	Ox	Sheep	Rabbit	Guinea pig	Rat	Mouse
ELAPIDAE											
ELAPINAE											
Bungarus coeruleus	Bombay	100	100	75	85	75	55	100	70	80	60
Naja naja	?	110	100	85	95	85	75	100	80	85	65
Micrurus frontalis	Butantan	5	105	65	100	5	25	60	80	100	85
Micrurus corallinus	"	80	80	65	80	100	75	85	110	65	80
VIPERIDAE											
VIPERINAE											
Vipera lebetina	?	105	105	75	85	80	75	100	80	75	65
Vipera russellii	?	105	105	75	85	80	65	105	80	80	60
Ecchis carinatus	Bombay	105	100	75	85	85	75	100	80	85	60
CROTALINAE											
Agkistrodon piscivorus	?	80	35	65	95	50	40	70	65	85	65
Trimeresurus flavoviridis	?	105	100	85	95	90	75	100	90	95	70
Lachesis muta muta	Butantan	80	100	70	80	70	60	75	75	75	60
Crotalus durissus terrificus (yellow venom)	"	105	90	65	90	85	60	100	85	85	70
Crotalus durissus terrificus (white venom)	"	105	90	75	95	80	65	100	90	90	70
Crotalus durissus durissus	Costa Rica	95	100	70	85	80	65	100	80	85	65
Bothrops jararaca	Butantan	65	100	70	80	70	65	85	75	75	65
Bothrops atrox	"	105	100	70	85	75	65	100	75	75	60
Bothrops cotiara	"	0	5	65	85	0	0	0	35	80	60
Bothrops itapetiningae	"	95	60	90	105	100	80	95	90	90	75
Bothrops jararacussu	"	80	100	70	85	80	60	85	75	80	60
Sensitivity — Mean Hemolysis		85	88	73	89	72	60	87	79	83	66

SPIDER VENOMS — In contrast to the snake venoms these were only exceptionally hemolytic, showing a specific action on certain types of erythrocytes. In the *Theraphosidae* family all tested venoms hemolyzed mouse cells, even when belonging to different sub-family or genus. Two of them, *Acanthoscurria atrox* and *Pamphobeteus roseus* also hemolyzed rat cells with smaller intensity. All other erythrocytes were resistant to these venoms.

Venom of *Phoneutria fera*, belonging to the *Ctenidae* family, was not able to hemolyze any of the 10 erythrocyte species.

From the *Lycosidae*, the venom of *Lycosa erythrognata* demonstrated variations of activity in relation to the erythrocyte species. It hemolyzed strongly sheep and ox red cells and less markedly human erythrocytes. It did not hemolyze erythrocytes from other species.

Venom of *Loxosceles rufipes*, family *Sicariidae*, had a specific and high hemolytic activity on human cells, but hemolyzed weakly ox cells and did not have any action on the others.

SCORPION VENOMS — The venoms of the two species tried, *Tityus bahiensis* and *Tityus serrulatus* belonging to the genus usually found in Brazil, were not hemolytic to any of the experimented erythrocyte species.

TABLE 2 — Percentage of hemolysis provoked by venoms on red blood cells of different species.
1 mg of venom — 20 x 10^6 red blood cells

SPIDERS	Venom origin	Human	Rhesus	Dog	Horse	Ox	Sheep	Rabbit	Guinea pig	Rat	Mouse
THERAPHOSIDAE											
THERAPHOSINAE											
Acanthoscurria atrox	Butantan	0	0	0	0	0	0	0	0	10	40
Acanthoscurria sternalis	"	0	0	0	0	0	0	0	0	0	25
Pamphobeteus roseus	"	0	0	0	0	0	0	0	0	15	60
Pamphobeteus sorocabae	"	0	0	0	0	0	0	0	0	0	50
Pamphobeteus tetracanthus	"	0	0	0	0	0	0	0	0	0	60
GRAMMOSTOLINAE											
Pterinopelma vellutinum	"	0	0	0	0	0	0	0	0	0	65
CTENIDAE											
CTENINAE											
Phoneutria fera	"	0	0	0	0	0	5	0	0	0	0
LYCOSIDAE											
LYCOSINAE											
Lycosa erythrognata	"	10	5	0	0	100	65	0	0	0	0
SICARIIDAE											
SICARIINAE											
Loxosceles rufipes	"	100	0	—	0	15	0	0	0	—	—
Loxosceles rufescens	"	—	—	0	—	—	—	—	—	0	0
SCORPIONS											
BUTHIDAE											
CENTURINAE											
Tityus bahiensis	Butantan	0	0	0	0	0	0	0	0	0	0
Tityus serrulatus	"	0	0	0	0	0	0	0	0	0	0

STORED SERUM — Table 3 contains data of determinations made with venoms of *Micrurus frontalis*, *B. cotiara*, *L. rufipes* and *L. erythrognata* which have shown specificity of hemolytic activity on different erythrocytes, and venoms of *L. rufescens*, *P. fera* and *B. jararaca*. A pool of horse sera stored for a long time has been used as supplier of lecithin. This serum had been kept at —15°C for about 40 days, and afterwards at +4°C for about 65 days. It was centrifuged before it was used in order to eliminate precipitates. With this serum, venom of *M. frontalis* hemolyzed human and ox cells which were resistant to this hemolysin. The action of the other snake venoms with this serum was practically the same. However, spider venoms, as the one of *Loxosceles rufipes*, specifically hemolytic to human erythrocytes, had lost its activity. The same happened with the venom of *Lycosa erythrognata* which in presence of the stored serum did not hemolyze human, ox and sheep red cells.

TABLE 3 — Comparison between percentage of hemolysis provoked by venom in presence of fresh serum (24 hours) and stored serum (40 days at — 15°C and 65 days a + 4°C).

1 mg of venom — 20 x 10^6 red blood cells.

VENOM	Serum	Human	Rhesus	Dog	Horse	Ox	Sheep	Rabbit	Guinea pig	Rat	Mouse
Micrurus frontalis	Fresh	5	105	65	100	5	25	60	80	100	85
	Stored	110	—	—	80	50	20	—	—	—	—
Bothrops cotiara	Fresh	0	5	65	85	0	0	0	35	80	60
	Stored	0	0	—	70	0	0	0	—	—	—
Bothrops jararaca	Fresh	65	100	70	80	70	65	85	75	75	65
	Stored	20	65	50	75	75	65	50	80	40	45
Loxosceles rufipes	Fresh	100	0	—	0	15	0	0	0	—	—
	Stored	0	—	0	—	0	—	0	—	0	0
Loxosceles rufescens	Fresh	—	—	0	—	—	—	—	—	0	0
	Stored	0	0	—	0	0	0	0	0	—	—
Lycosa erythrognata	Fresh	10	5	0	0	100	65	0	0	0	0
	Stored	20	—	—	0	0	0	—	—	—	—
Phoneutria fera	Fresh	0	0	0	0	0	5	0	0	0	0
	Stored	0	—	—	—	—	0	—	—	—	—

A comparison was done between the hemolytic activity of *M. frontalis* and *B. jararaca* venoms on human red cells, using stored and fresh sera (table 4). The results obtained confirmed what has been previously observed, *i.e.*, human erythrocytes were hemolyzed by the venom of *M. frontalis* when in presence of stored serum but no hemolysis occured in presence of fresh serum. On the other hand, hemolytic action of *B. jararaca* venom remained unaffected. An estimation of lipids and cholesterol content in both sera (table 4) showed loss of cholesterol in the stored serum which was probably eliminated by centrifugation, after being precipitated by storage.

TABLE 4 — Hemolysis of human red cells (percentage) by snake venom in presence of preserved horse serum (40 days at — 15°C and 65 days at + 4°C) and of fresh serum Total lipids and cholesterol contents of sera

Serum age	Hemolysis %		Total lipids g %	Cholesterol mg %	Lipids without cholesterol g %
	Bothrops jararaca	Micrurus frontalis			
3 1/2 months....	75	110	1,117	2,0	1,115
24 hours	70	0	1,224	113,0	1,111

DISCUSSION

The hemolytic activity of different venoms was determined as concerns their indirect action, *i.c.*, in presence of serum lecithin for lecithinase formation as hemolytic agent, since it has been demonstrated by Rosenfeld, Kelen and Nudel, (17) that no one of the experimented venoms, with exception of bee venoms (*Apis mellifica*), was able to hemolyze washed sheep erythrocytes directly (17).

As for the relation between venom hemolytic activity and the zoological classification of the snakes, it has been observed that different genus of one sub-family, *Bungarus* and *Naja* of sub-family *Elapinae*, had similar activities. The same was observed for different species of one genus, *Vipera*, species *lebetina* and *russellii*, or the *Crotalus* genus. However, sometimes one genus did not have hemolytic activity on some erythrocytes (*M. frontalis*) while there was similarity of activity among other genus belonging to the same sub-family (*Bungarus, Naja*). The same exception was observed for the species *cotiara* of *Bothrops* genus in contrast to the other *Bothrops* species that had similar activity on the different erythrocytes.

The lack of correlation between hemolytic activity and zoological classification is also evidenced in scorpion venoms, among which, the two venoms experimented, *T. bahiensis* and *T. serrulatus* did not have hemolytic activity on any of the red cells used. Houssay in 1919 (6) also observed that the venom of *T. bahiensis* was not hemolytic, as well as that of *Buthus quinquestriatus*, both belonging to family *Buthidae*, and this lack of activity was also stated by Balozet (1,2) with venoms of *Androctonus australis* and *Buthus occitanus* of the same family, on horse erythrocytes. On the other hand, venom of *Buthacus arenicola* also belonging to family *Buthidae* hemolyzed horse red cells, and this activity was also observed in venom of *Scorpio maurus*, family *Scorpionidae*.

The sensitivity of each erythrocyte species to hemolysis, by all venoms, indicated mouse and sheep erythrocytes as the more resistant species with 66% and 59% mean hemolysis respectively, and horse cells are the more sensitive ones, with

89%. This sensitivity degree, however, is not an absolute value as resistant erythrocytes, as those of mouse were hemolyzed by all snake venoms, while another resistant species as sheep erythrocytes, were not hemolyzed by *B. cotiara* venom. On the other hand, very sensitive erythrocytes as the rhesus ones could be resistant to one venom (*B. cotiara*) while the very sensitive horse erythrocytes were hemolyzed by all venoms.

All spider venoms of family *Theraphosidae* (bird spiders) hemolyzed mouse corpuscles while none of the other spider venoms had any action on them. These spiders, precisely, had been nourished with new-born mouses, which seems to be also their nourishment in natural conditions. This fact is stated without trying to give any explanation to such a coincidence, but it is a matter for further investigations.

The very specific action of some venoms on some erythrocytes species suggests a sensitivity of those animals to such venoms. Yet this is not confirmed by the clinical symptoms observed in accidents provoked by some of these poisonous animals. There is no appreciable hemolysis in humans bitten by snakes of *Bothrops* genus, while the hemolysis is intense in *Crotalus durissus terrificus* bitten individuals. However, the hemolytic activity of these venoms "in vitro" is almost alike. In the case of *Loxosceles rufipes* venom, it seems to exist a concordance between clinical data and the erythrocytes sensitivity. Thus, this venom which is extremely toxic to man by provoking intense hemolysis leading to severe anemia, ieterus and in some cases even death, is also extremely hemolytic to human erythrocytes "in vitro". This venom is a weak toxin for mice and does not hemolyze their erythrocytes "in vitro".

The fact that the venom of *Micrurus frontalis* hemolyzed human red cells with intensity when stored serum was used, and this hemolysis did not take place with fresh serum (table 3), suggested that an inhibitory substance to hemolysis could have been disappeared. Since the inhibitory effect of cholesterol is already known, both in hemolysis provoked by venom (5) and in hemolysis provoked by saponin or other chemical reagents, as it was demonstrated by Ponder (16), an estimation of the cholesterol concentration in the sera was carried out. In fact this estimation confirmed the absence of this lipid in the stored serum. Thus the disappearence of cholesterol seems to have favoured the hemolysis action of *M. frontalis* which could not act in the presence of this substance on human and ox erythrocytes. On the other hand venoms of *Loxosceles rufipes* and *Lycosa erythrognata* which had been specifically active on some of the red cells species in presence of cholesterol, did not have hemolytic activity anymore when the serum laeking cholesterol was used. On the other hand, *B. jararaca* venom had a decreased activity with stored serum, while the effect produced by *B. cotiara* venom remained the same. *I.c.*, cholesterol inhibits some venoms in relation to some erythrocyte species, but can activate others.

This fact, the variation in degree of hemolysis produced by one venom in relation to different erythrocyte species as well as the finding that one venom may hemolyze some species of erythrocyte while being inactive toward others, demonstrate the existence of different hemolytic substances in the various venoms and the plurality of factors with such effect in one same venom.

The results obtained show clearly that the hemolytic activity of one venom cannot be defined without mentioning the erythrocyte species used for its determination. For instance, venom of *M. frontalis* if tried on human red cells would be considered as non-hemolytic, when in relation to dog or horse erythrocytes it would be defined as extremely active. The same would happen to venom of *B. cotiara*. This discrepancy is stronger for spider venoms, the one of *Loxosceles rufipes* would be described as extremely hemolytic when determined its activity in relation to human erythrocytes, but with any of the other species it would be considered as being inactive.

SUMMARY

Sensitivity of human, rhesus, dog, horse, ox, sheep, rabbit, guinea-pig, rat and mouse erythrocytes to the hemolytic activity of some animal venoms (17 snakes, 10 spiders, and 2 scorpions) was investigated. Indirect hemolytic activity of venoms was determined on erythrocytes by estimating colorimetrically the liberated hemoglobin.

No correlation was found between the hemolytic activity of the venoms and the zoological clasification of the poisonous animals, for the different erythrocytes used.

Snake venoms, in general, hemolyzed all 10 erythrocyte species. The only exceptions were the venom of *Micrurus frontalis*, which did not hemolyze human and ox erythrocytes, and that of *Bothrops cotiara* which did not hemolyze those cells as well as the erythrocytes of rhesus, sheep and rabbit.

Spider venoms of *Theraphosidae* family hemolyzed only mouse red cells, and some of them also rat cells. The venom of *Loxosceles* hemolyzed markedly human corpuscles and weakly those of ox. *Lycosa* venom lyzed ox and sheep erythrocytes and weakly human erythrocytes. The venom of *Phoneutria* was not hemolytic. All other spider venoms were non hemolytic towards all types of erythrocytes.

Venoms of *Tityus bahiensis* and *Tityus serrulatus*, the two scorpions most frequently found in Brazil, were not hemolytic to anyone of the erythrocyte species.

The sensitivity degree of each erythrocyte species was calculated to the hemolysis by all venoms, but it was observed that very sensitive erythrocytes could be resistant to certain venoms and vice-versa.

The absence of cholesterol in a serum used as lecithin supplier can activate hemolysis by some venoms, but, on the other hand, it can inhibit activity of others.

There is specificity of erythrocytes sensitivity to each venom, demonstrating the complexity of hemolytic activity that must be the effect of various factors. Therefore it is necessary to indicate the species of the erythrocyte used when defining the hemolytic activity of animal venoms.

RESUMO

Foi verificada a sensibilidade de hemácias humanas, de rhesus, cão, cavalo, boi, carneiro, coelho, cobaia, rato e camundongo à hemolisina de alguns venenos animais (17 ofídicos, 10 aracnídicos e 2 escorpiônicos), tendo se determinado a atividade hemolítica indireta dos venenos sôbre os glóbulos, dosando a hemoglobina liberada por método colorimétrico.

Não houve relação entre a ação hemolítica dos venenos e a classificação zoológica dos animais peçonhentos para os diferentes glóbulos experimentados.

Os venenos ofídicos de modo geral hemolisaram tôdas as 10 espécies de glóbulos, fazendo exceção apenas os venenos de *Micrurus frontalis* que não hemolisou glóbulos humanos e de boi, e *Bothrops cotiara* que além destes não hemolisou os de rhesus, carneiro e coelho. Os venenos de aranha da família *Theraphosidae* hemolisaram sòmente glóbulos de camundongo, alguns também os de rato. O de *Loxosceles* hemolisou intensamente glóbulos humanos e um pouco os de boi. O de *Lycosa* lisou hemácias de boi e carneiro e um poueo os humanos. O veneno de *Phoneutria* não foi hemolítico. Todos êles foram inativos para as outras hemácias não referidas.

Venenos de *Tityus bahiensis* e *Tityus serrulatus*, os dois escorpiões mais eomumente encontrados no Brasil, não foram hemolíticos para nenhuma espécie de hemáeia.

Foi caleulado o gráu de sensibilidade média de cada hemácia à hemólise com todos os venenos, mas foi verifieado que glóbulos muito sensíveis podem deixar de ser lisados por certos venenos e vieeversa.

A ausência de colesterol no sôro empregado como fonte de lecitina pode ativar a hemólise de certos venenos, porém, por outro lado, pode inibir a atividade de outros.

Há especificidade na sensibilidade dos glóbulos para cada veneno, demonstrando haver complexidade na atividade hemolítica que deve ser efeito de vários fatores. Por outro lado, evidencia-se a necessidade de mencionar a espécie de hemáeia quando se quer definir a atividade hemolítica de venenos.

Acknowledgements. Our thanks are due to Dr. Deoras, Haffkine Institute, Bombay, India, who provided the samples of *Bungarus caeruleus* and *Echis carinatus*. We

are also in debt to Dr. W. Bücherl, Department of Medical Zoology, Instituto Butantan, who supplied the samples of scorpion and spider venoms used in this work.

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